

# Differential effects of bacterial lipopolysaccharides upon neutrophil function

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**Abstract** Lipopolysaccharide (LPS) is a potent inflammatory agent which augments neutrophil sensitivity to subsequent inflammatory stimuli. In this study, the effects of structurally different LPS types upon neutrophil effector functions were examined. Rough LPS types, which have lost the *O*-polysaccharide moiety, were found to act more rapidly than smooth LPS types in stimulating neutrophil  $\beta_2$  integrin activity and fMLP-induced respiratory burst. These findings suggest an involvement of the *O*-polysaccharide region of LPS in regulating neutrophil responsiveness to different LPS chemotypes with important implications for the mechanisms underlying regulation of the inflammatory response in conditions associated with elevation of LPS in plasma, e.g. septic shock or acute respiratory distress syndrome.

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**Key words:** Inflammation; Neutrophil; Endotoxin

## 1. Introduction

Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is a potent inflammatory mediator which involves production of pro-inflammatory cytokines and induces functional alterations in many cell types. These effects contribute to the generation of clinical symptoms associated with septic shock [1] which may lead to development of disease states such as acute respiratory distress syndrome [2,3]. LPS can increase neutrophil sensitivity to subsequent inflammatory stimuli by 'priming' them for augmented production of oxygen metabolites and secretion of granule contents [4,5]. In addition, LPS can augment neutrophil adhesiveness [6] which could result in an accumulation of neutrophils in the vasculature of the lung and other organs. Although these functional alterations induced by LPS are principally directed towards increased efficiency of bacterial clearance, under certain conditions, neutrophil-mediated tissue damage may occur, thus contributing to disease pathogenesis.

The efficiency of LPS-mediated effects is influenced by serum components, including the well-characterised LPS bind-

ing protein (LBP) [7]. Several pathways for LPS-induced neutrophil activation have been proposed [8,9]. One pathway involves the formation of a membrane (m) CD14 (a glycosyl phosphatidyl inositol (GPI)-linked receptor for LPS)-CR3 complex [10]. Alternatively, interaction of LPS with either mCD14 [11] or another distinct receptor [12] has been suggested to mobilise putative signal-transducing molecules. Recent evidence suggests that the  $\beta_2$  integrin CR3 could function directly as an LPS signalling receptor [13]. Another pathway has been suggested to involve soluble (s) CD14-LPS complexes binding a novel receptor at the surface of cells which lack mCD14 (e.g. non-myeloid cell types or cells from paroxysmal nocturnal haemoglobinuria patients which lack GPI-linked receptors).

Most types of LPS are composed of three distinct regions: the hydrophilic *O*-polysaccharide moiety of heterogeneous length, the core oligosaccharide and the hydrophobic domain known as lipid A. The lipid A moiety of LPS contains the region of the LPS molecule which confers its ability to affect neutrophil function and this structure is highly conserved among diverse species of Gram-negative bacteria [14]. Neutrophils are apparently especially discriminating with respect to the chemical structure of lipid A. Deacylated LPS is not active in priming neutrophils [15] and monophosphoryl lipid A does not prime neutrophils, but does prime monocytes [16]. Thus, chemical modification of lipid A by bacteria may confer protection against host defence mechanisms. In keeping with this suggestion, most enterobacteria, like *Escherichia coli*, produce LPS which has a hydrophilic *O*-polysaccharide moiety, a core oligosaccharide and the hydrophobic lipid A domain and is termed 'smooth' or 'S-form' chemotype. Loss of the *O*-polysaccharide region results in LPS mutants of enterobacteria described as 'rough' or the 'R-form' chemotype of LPS.

In this present study, we sought to investigate the mechanisms of LPS action in priming neutrophil function by examining the effect of smooth and rough chemotypes of LPS upon neutrophil function. Here we report our detailed analysis demonstrating that rough LPS (rLPS) acts more rapidly than smooth LPS (sLPS) to cause functional alterations in neutrophils. Activation of  $\beta_2$  integrin-mediated adhesion occurred within 15 min following rLPS treatment whereas sLPS was without effect at this early time point. Rough LPS also primed neutrophil formyl-Met-Leu-Phe (fMLP)-induced respiratory burst activity more rapidly. These findings suggest that the *O*-polysaccharide moiety of LPS can profoundly influence the responsiveness of neutrophils to LPS with important consequences for initiation and progression of the inflammatory response.

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**Abbreviations:** LPS, lipopolysaccharide; LBP, lipopolysaccharide binding protein; TNF, tumour necrosis factor; fMLP, formyl-Met-Leu-Phe; PAF, platelet activating factor; ACLB, albumin-coated latex beads

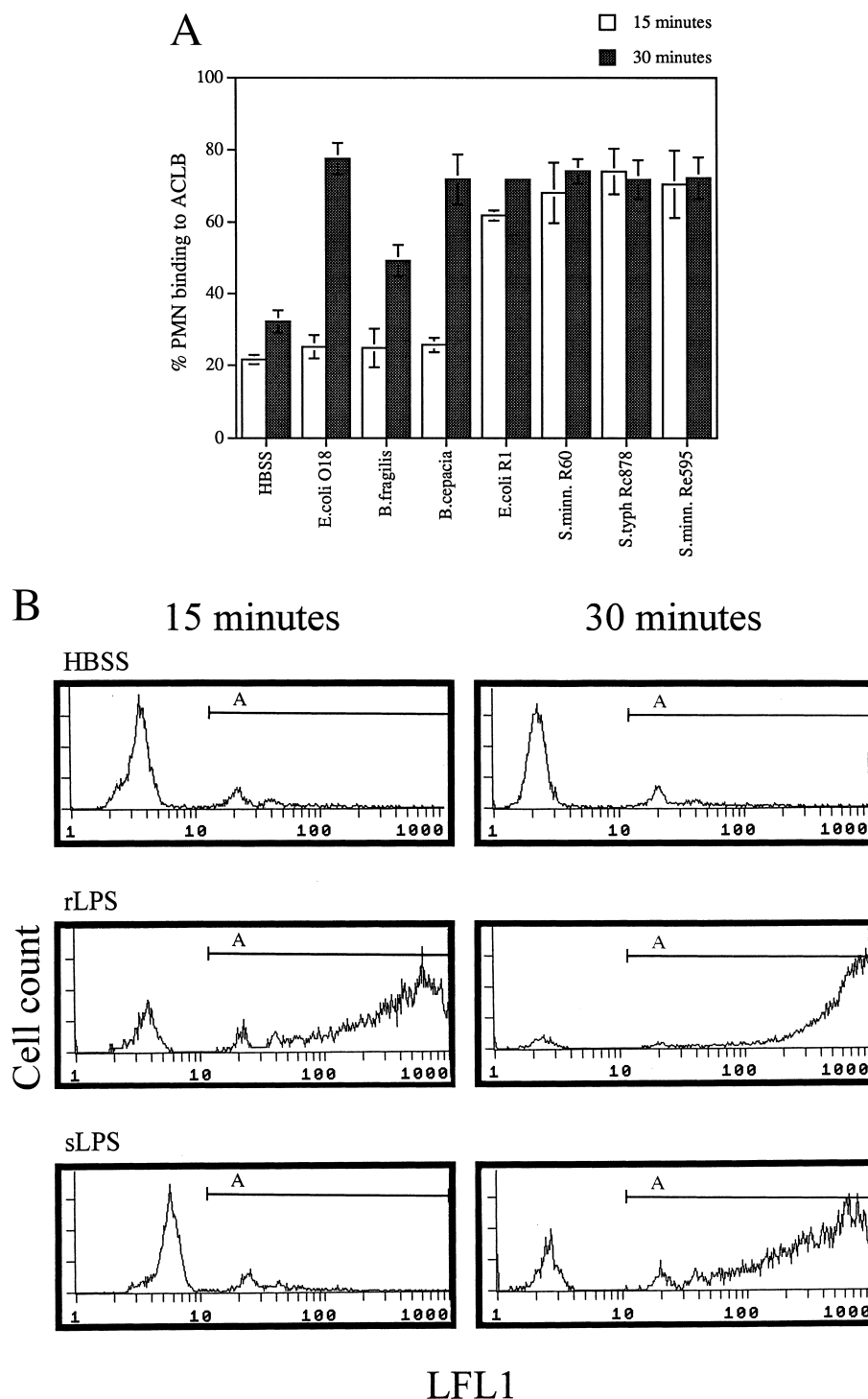


Fig. 1. Effect of various LPS chemotypes upon neutrophil  $\beta_2$  integrin-dependent binding. A: Freshly isolated neutrophils ( $10^7/\text{ml}$ ) were incubated at  $37^\circ\text{C}$  with  $500 \text{ ng/ml}$  LPS for 15 or 30 min in the presence of ACLB and binding assessed by flow cytometry. Data represent the mean percentage of neutrophils binding one or more ACLB  $\pm$  S.E.M. of three different experiments. B: Histograms of a representative experiment showing flow cytometric determination of neutrophil binding to fluorescent ACLB in presence of rough (*E. coli* R1) or smooth LPS (*E. coli* O18). Gate A represents one or more fluorescent beads bound to neutrophils.

## 2. Materials and methods

### 2.1. Antibodies and other reagents

Dextran T500 was obtained from Pharmacia Biotech Ltd (Milton Keynes, UK). Hanks' balanced salt solution (HBSS) was obtained from Life Technologies (Paisley, UK). Human serum albumin, fMLP peptide, cytochrome *c* and superoxide dismutase were obtained

from Sigma Chemical Co. (Poole, UK). Dihydrorhodamine (DHR 123) was purchased from Molecular Probes, Eugene, OR, USA (supplied by Cambridge Bioscience, Cambridge, UK). Fluorescent latex beads ( $1 \mu\text{m}$  diameter) were from Polysciences Inc., Warrington, PA, USA (supplied by Park Scientific, Northampton, UK). FITC-conjugated  $\text{F(ab')}_2$  goat anti-mouse immunoglobulins were from DAKO. Mouse mAb ICRF44 (CD11b) (IgG1) was obtained from Dr Nancy Hogg and Leu-8 (CD62L) (IgG2b) was obtained from Becton Dick-

inson, High Wycombe, UK. All antibodies were used at a concentration that saturated binding as assessed by flow cytometric analysis.

## 2.2. Neutrophil isolation

Polymorphonuclear leukocytes were isolated from peripheral blood of healthy donors by dextran sedimentation and fractionation through isotonic discontinuous Percoll gradients as previously described [17]. Polymorphonuclear leukocytes were found to be 95–98% neutrophils by morphological criteria and viability was always >99% as assessed by trypan blue exclusion. Autologous platelet-depleted plasma was prepared by centrifugation of platelet-rich plasma at  $13\,000\times g$  for 5 min.

## 2.3. Bacterial lipopolysaccharides

The different LPS types used in this study were extracted from lyophilised bacteria by either the aqueous phenol method for smooth chemotypes or petroleum/chloroform/phenol for rough chemotypes [18]. Smooth LPS were from *Escherichia coli* O18, *Bacterioides fragilis* NCTC9343 and *Burkholderia cepacia* and rough LPS from *E. coli* R1 and R3 and *Salmonella minnesota* R60 or Re595 and *Salmonella typhimurium* Re878. Stock solutions (1 mg/ml) in water were sonicated and then stored at  $-20^{\circ}\text{C}$ . LPS from *E. coli* O111 was obtained from Sigma (Poole, UK). Purified LPS types were sonicated for 2 min to ensure uniform resuspension prior to final dilution in HBSS for the experiments.

## 2.4. Measurement of neutrophil shape change and adhesion to albumin-coated latex beads

The effects of different LPS types upon neutrophil shape change was determined by flow cytometric analysis as previously described [19] and verified by conventional light microscopic analysis of samples.

CD11b/CD18-dependent binding of albumin-coated latex beads (ACLB) to neutrophils in the presence or absence of LPS was measured as previously described [19]. Both assays were performed in the presence of 1% autologous platelet-depleted plasma.

## 2.5. Indirect immunofluorescence flow cytometry

Neutrophils ( $10^5$  cells per test) were incubated with 50  $\mu\text{l}$  of saturating concentrations of mAb for 30 min at  $4^{\circ}\text{C}$ , then washed three times with HBSS containing 0.2% bovine serum albumin (BSA) and 0.1% sodium azide to remove unbound mAb and incubated with 50  $\mu\text{l}$  of FITC-conjugated F(ab')<sub>2</sub> goat anti-mouse Ig (1:40) for 30 min at  $4^{\circ}\text{C}$ . Neutrophils were then washed three times with HBSS/BSA/azide prior to analysis by flow cytometry using a Coulter Epics Profile II cytometer (Coulter Electronics, Luton, UK).

## 2.6. Measurement of endogenous oxidant production and superoxide anion release

Production of reactive oxygen intermediates by neutrophils with and without exposure to LPS was assessed by flow cytometric determination of DHR 123 oxidation as previously described [19] in the presence of 1% autologous platelet-poor plasma.

For determination of fMLP-induced release of superoxide anions, neutrophils were pre-incubated with LPS in presence of 1% autologous plasma for 15 or 30 min followed by addition of HBSS or fMLP ( $10^{-7}$  M) and cytochrome *c* (1 mg/ml). Each assay was performed in triplicate and also in the presence of superoxide dismutase (200 U/ml) to confirm the specificity of cytochrome *c* reduction. After 15 min at  $37^{\circ}\text{C}$ , the reaction was terminated by placing the cells on ice, followed by centrifugation ( $13\,000\times g$ , 3 min,  $4^{\circ}\text{C}$ ). The superoxide dismutase inhibitable reduction of cytochrome *c* was determined for each supernatant by measuring the peak absorbance between 535 and 565 nm using a Pye-Unicam scanning spectrophotometer. Results are expressed as nanomoles of superoxide anions generated per  $10^6$  neutrophils calculated using the extinction coefficient of  $21\times 10^3\text{ M}^{-1}\text{ cm}^{-1}$ .

## 2.7. Statistical analysis

The mean with S.E.M. or S.D. was calculated from the means from different experiments (different donors), each performed in triplicate. Using Student's *t*-test (paired, one-tailed), the significance of the difference between the mean of the assays and control groups was determined. *P* values <0.05 were considered significant.

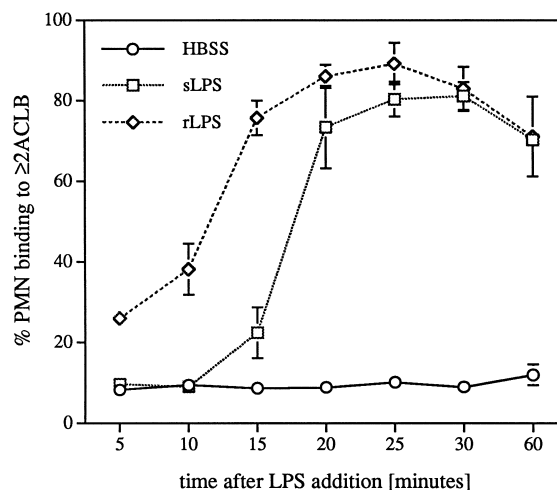


Fig. 2. Kinetics of effects of rough and smooth LPS on  $\beta_2$  integrin activity. Neutrophils were incubated at  $37^{\circ}\text{C}$  with ACLB and 500 ng/ml LPS (*E. coli* O111 or R3) was added at different time points indicated. Data represent the mean  $\pm$  S.E.M. of 3–6 experiments.

## 3. Results

### 3.1. Differential effects of LPS chemotypes upon neutrophil $\beta_2$ integrin activity

Altered expression and function of  $\beta_2$  integrins occurs rapidly following receptor-mediated activation. Our previous studies have shown that ACLB binding represents a sensitive index of CD11b/CD18 functional activity [19]. We therefore investigated the ability of different LPS chemotypes to induce neutrophil  $\beta_2$  integrin activity. The effect of LPS from seven different species of bacteria, three smooth forms (sLPS) and four rough forms (rLPS), on neutrophil binding to ACLB was determined. In preliminary experiments, we found that there was an absolute requirement for the presence of low concentrations of plasma for LPS-induced functional alterations in neutrophils (data not shown). Surprisingly, significant differences were observed in temporal analyses of the effects of LPS chemotypes. Although the majority of neutrophils treated with all LPS chemotypes for 30 min showed induction of CD11b/CD18 functional activity as determined by ACLB binding (Fig. 1A,B), a clear discrimination between the effects of smooth and rough LPS was seen after shorter pre-incubation times (Fig. 1 and data not shown). The observed differential effects of LPS chemotypes upon neutrophil  $\beta_2$  integrin function at 15 min were also paralleled by differences in the effects of rLPS and sLPS upon neutrophil polarisation as assessed by microscopic analysis and also the increase in forward angle laser scatter of neutrophils in flow cytometric analysis (data not shown).

We next performed a detailed examination of the time course of the effects of the different LPS chemotypes in inducing  $\beta_2$  integrin-dependent neutrophil binding to ACLB. Data shown in Fig. 2 indicate that exposure of neutrophils to rLPS rapidly induces  $\beta_2$  integrin activation (within 5 min), whereas sLPS effects were temporally delayed (within 20 min). These data indicated that rough LPS acted more rapidly to stimulate neutrophil function than smooth LPS.

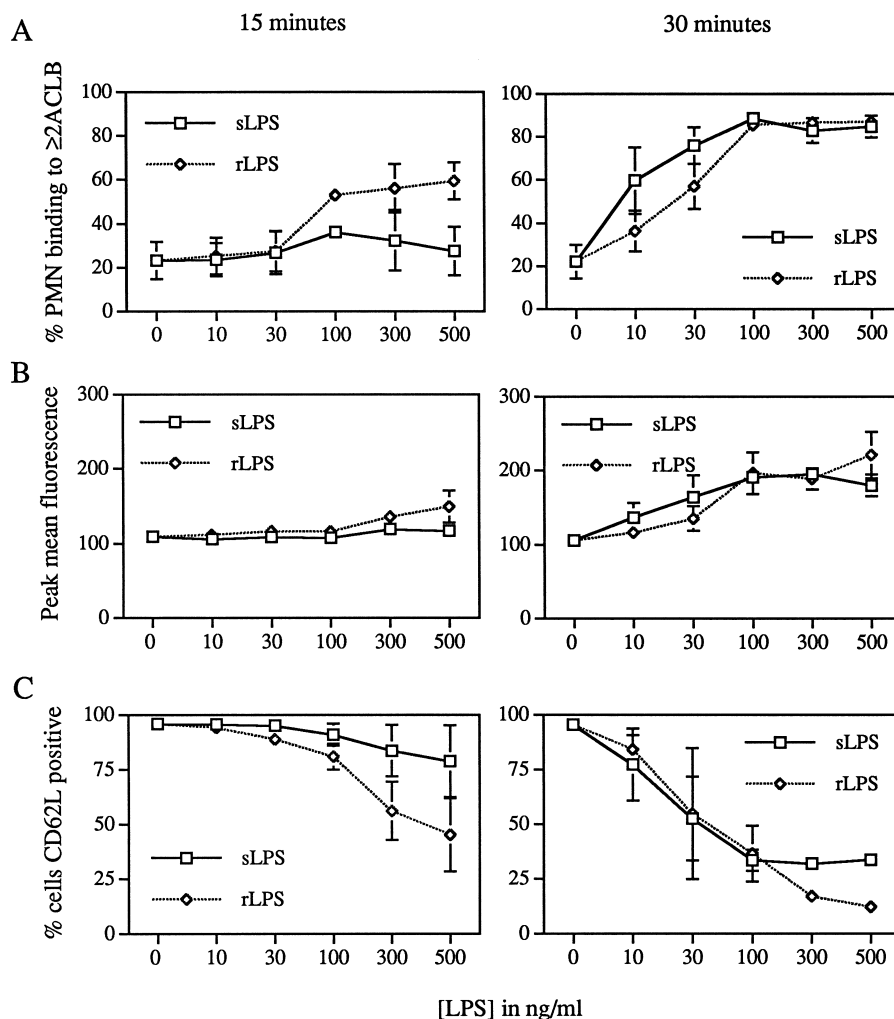


Fig. 3. The differential effect of rough and smooth LPS is not due to a difference in molecular weight. A: Neutrophils were incubated for 15 or 30 min at 37°C with different concentrations of rough (*E. coli* R3) or smooth LPS (*E. coli* O111) in the presence of ACLB. B: Neutrophil CD11b expression following incubation with rough or smooth LPS was assessed by flow cytometry using an anti-CD11b mAb (44 mAb). C: Neutrophil CD62L expression following incubation with rough or smooth LPS was assessed by flow cytometry using an anti-CD62L mAb (Leu-8 mAb). Data shown are the mean  $\pm$  S.E.M. of three experiments.

### 3.2. The differential effect of rLPS and sLPS is not due to a difference in molecular weight

The experiments described above compared equivalent weight/volume concentrations of LPS. The average molecular weight of sLPS is approximately four times more than that of rLPS, thus rLPS contains at least four times more of the active lipid A moiety than the equivalent weight of sLPS. We therefore sought to determine whether the differential effect of sLPS and rLPS was due to differences in molecular weight between rLPS and sLPS chemotypes.

We compared the concentration responses of rLPS and sLPS for induction of  $\beta_2$  integrin-dependent ACLB binding and also for alterations in expression of CD11b and CD62L. At 15 min, rLPS augmented  $\beta_2$  integrin functional activity at concentrations greater than 100 ng/ml (Fig. 3A). Expression of CD62L was reduced in a concentration dependent manner in response to rLPS (Fig. 3C), consistent with the suggestion that shedding of this receptor is a sensitive indicator of neutrophil activation [20]. In contrast, a small and variable effect of sLPS upon CD62L expression was observed

at 15 min which failed to reach statistical significance (Fig. 3C). Although expression of CD11b was increased at higher concentrations of rLPS (500 ng/ml) at 15 min, this failed to reach statistical significance representing an increase of less than 25% relative to untreated cells (Fig. 3B). Interestingly, induction of  $\beta_2$  integrin activity by rLPS occurred at concentrations which did not augment CD11b expression, suggesting that rLPS effects reflected changes in receptors already present on the neutrophil surface and not mobilisation of CD11b/CD18 from intracellular compartments. In marked contrast to the effects of rLPS, sLPS did not alter  $\beta_2$  integrin functional activity or CD11b expression (Fig. 3A,B). Whilst analysis of the effects of various concentrations of rough and smooth LPS types upon neutrophil  $\beta_2$  integrin activation at 15 min revealed clear differences, the effects of rough and smooth LPS types were indistinguishable at 30 min. These data strongly suggest that the differential effects of rough and smooth LPS upon neutrophil  $\beta_2$  integrin activation cannot be simply explained by differences in molecular weight.

### 3.3. Smooth LPS does not compete with rLPS in neutrophil binding to ACLB

We next considered the possibility that differential glycosylation of smooth LPS might fail to initiate signalling pathways despite effective binding to the neutrophil surface. To test this suggestion, we used a low concentration of rLPS (100 ng/ml) which stimulated  $\beta_2$  integrin function, in the presence of increasing concentrations of sLPS. Consistent with data shown in Fig. 1, high concentrations (500 and 1000 ng/ml) of sLPS did not affect  $\beta_2$  integrin activity at 10 min, but caused maximal activation at 30 min (Fig. 4: compare HBSS+sLPS for both time points). Thus, the sLPS used in these experiments was capable of causing functional alterations in neutrophils. However, despite the observation that sLPS was able to bind and initiate signals which augment  $\beta_2$  integrin activity at 30 min, the presence of high concentrations of sLPS did not interfere with the ability of low concentrations of rLPS to rapidly augment  $\beta_2$  integrin ligand binding (Fig. 4). These data raise the possibility that either sLPS binds more slowly to the neutrophil surface than rLPS, or that the receptors which mediate responses to rLPS and sLPS are different.

### 3.4. Neutrophil respiratory burst induced by LPS

In order to investigate whether sLPS and rLPS exerted differential effects upon other neutrophil effector functions, we next looked at the induction of neutrophil respiratory burst activity assessed by changes in fluorescence of DHR123 due to oxidant generation. As shown in Fig. 5A, in absence of fMLP stimulation, only rLPS was effective in altering intracellular oxidant species production in neutrophils at 15 min in the presence of 1% autologous platelet-poor plasma. It should be noted that rLPS failed to induce changes in DHR123 fluorescence in the absence of plasma (data not shown).

To extend these observations, fMLP-induced superoxide anion release was measured following LPS pre-incubation

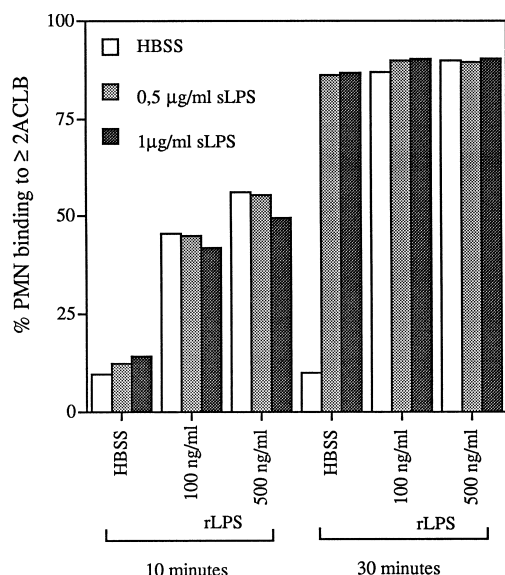


Fig. 4. Smooth LPS does not block rough LPS-induced ACLB binding. Neutrophils were incubated in the presence of ACLB for 10 or 30 min at 37°C with 100 or 500 ng/ml rLPS (*E. coli* R3) in the presence of different concentrations of sLPS (*E. coli* O111) as indicated. Data shown are from one representative experiment of two that were performed.

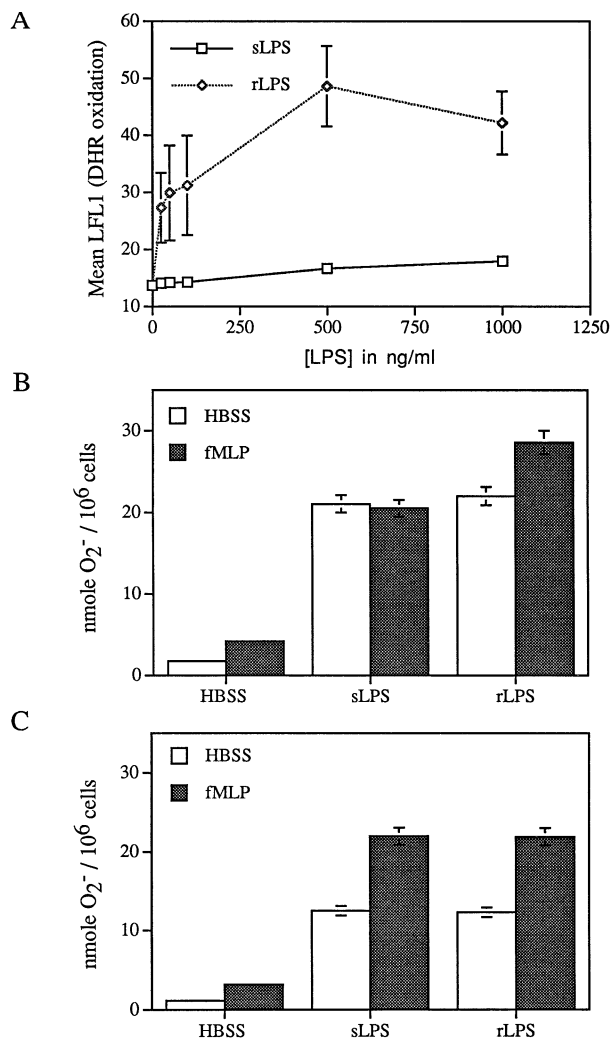


Fig. 5. Neutrophil respiratory burst induced by LPS. A: Oxidant production of neutrophils incubated with rough (*E. coli* R1) or smooth LPS (*E. coli* O18) in the absence of fMLP for 15 min at 37°C was determined by measurement of changes in fluorescence of DHR123. B and C: Superoxide anion release was determined by measuring the superoxide dismutase-inhibitable reduction of cytochrome *c* from neutrophils pre-incubated with rough LPS (*E. coli* R1) or smooth LPS (*E. coli* O18) at 500 ng/ml for 15 min (B) or 30 min (C) followed by addition of HBSS or fMLP ( $10^{-7}$  M). Data presented here are mean  $\pm$  S.D. of three experiments.

with rough and smooth LPS at 500 ng/ml in the presence of 1% autologous plasma. The release of superoxide anions was low in unstimulated cells and addition of fMLP ( $10^{-7}$  M) alone caused a small increase in superoxide release (Fig. 5B,C), confirming that these neutrophils were not 'primed'. Neither rough or smooth LPS chemotypes analysed here induced superoxide anion release when plasma was omitted from the assay (data not shown). However, in the presence of 1% plasma, both rough and smooth LPS chemotypes at 500 ng/ml caused a marked increase in superoxide anion release at both 15 and 30 min, possibly reflecting the relatively high concentrations used. It is interesting to note that following 15 min pre-incubation with sLPS superoxide release was not further augmented by fMLP, whereas a small but significant increase in fMLP-induced superoxide release was observed for neutrophils treated with rLPS for 15 min (Fig.

5B). In contrast, at 30 min fMLP-induced release was further augmented following pre-incubation with both LPS chemotypes (Fig. 5C). Considering all the effects of sLPS upon neutrophil function at 15 min, although polarisation,  $\beta_2$  integrin-mediated adhesion and intracellular production of oxidant species were not affected by sLPS treatment, superoxide anion release was augmented. One interpretation of these data might be that sLPS might initiate two signals, a rapid direct augmentation of superoxide release and a slower 'priming' effect associated with polarisation and increased  $\beta_2$  integrin activity and augmentation of fMLP-induced superoxide release. Whilst these data clearly show both rough and smooth LPS chemotypes induce functional changes in neutrophils, our interpretation of these findings is that rLPS acts more rapidly to induce 'priming' effects.

#### 4. Discussion

Regulation of neutrophil adhesiveness is generally considered to be a key element in the development of inflammatory reactions. The presence of LPS in inflammatory responses has been linked to the pathogenesis of diseases in which neutrophil-mediated tissue damage occurs [2]. In this study we have examined the effect of different types of LPS upon neutrophil  $\beta_2$  integrin activity and production of potentially injurious reactive oxygen species. Rough LPS types were found to induce alterations in  $\beta_2$  integrin activation, CD62L shedding and intracellular production of oxidant species more rapidly than smooth LPS types, suggesting a possible role for carbohydrate modification of LPS in regulation of neutrophil activation.

$\beta_2$  integrin function of neutrophils treated with the different types of LPS was examined using a flow cytometric assay that measures neutrophil binding to ACLB [6,19]. Although LPS has been suggested to bind to a number of serum proteins including albumin, it is unlikely that LPS bind directly to ACLB 'opsonising' them for binding through 'LPS receptors' since no ACLB were bound to neutrophils in absence of plasma or serum, even at high concentration of LPS (data not shown). Furthermore, few ACLB were bound by unstimulated neutrophils and CD18 mAb effectively blocked LPS-induced ACLB binding (Fig. 1 and data not shown). In the presence of low concentrations of plasma, maximum binding was induced by all types of LPS examined here within 30 min, but a differential effect of smooth and rough LPS was observed at earlier time points. Dose responses of LPS clearly indicated that observed differential effects were not due to differences in molecular weight of LPS chemotypes and therefore may reflect altered responses of neutrophils to carbohydrate modification of LPS.

The key role of CD14 in responses to LPS stimulation has been extensively demonstrated [21]. LBP/soluble CD14 complexes present in serum may facilitate response to LPS by accelerating LPS binding to CD14. A site-specific mutation in CD14 showed selective binding and transfer of *E. coli* LPS but not *Porphyromonas gingivalis* LPS suggesting specificity of binding and transfer of different LPS molecules via the CD14 pathway [22]. Although neutrophil responses to LPS/sCD14 and LPS/LBP complexes are thought to be similar, rough LPS effects have been suggested to be largely independent of formation of complexes with sCD14 [9]. We found a requirement for the presence of serum components for LPS-

induced functional alterations, consistent with a role for LBP or sCD14. Interestingly, although both rough and smooth LPS chemotypes induced release of superoxide from neutrophils after 15 min, a clear differential effect was seen upon polarisation, adhesion, intracellular production of oxidant species and augmentation of fMLP-induced superoxide release, with sLPS being relatively ineffective at 15 min. Both LPS chemotypes caused a similar degree of functional activation at 30 min, implying that both sLPS and rLPS can bind neutrophils and induce intracellular signals. Our data relating to LPS-induced changes in  $\beta_2$  integrin-mediated ACLB binding, DHR123 fluorescence, and fMLP-induced superoxide release would be consistent with a model in which carbohydrate modification of LPS may attenuate transfer of LPS from LBP to LPS receptors on the neutrophil surface. The lack of differential effect of LPS chemotypes upon unstimulated superoxide release at 15 min is interesting in that it raises the possibility that glycosylation of LPS may influence events which may be associated with 'priming'.

Recent data indicate that cellular recognition and subsequent responses to LPS may be more complex than previously thought. Although CD14 was shown to play an important part in LPS responses in presence of LBP [23], a CD14-independent LPS signalling pathway is now accepted [24,25]. It has been recently proposed that other neutrophil receptors may bind LPS and initiate intracellular signalling cascades. L-selectin has been shown to bind to LPS and activate neutrophil effector function [26], which may explain why L-selectin-deficient mice are resistant to high doses of LPS. Furthermore, differential binding of LPS from various strains of bacteria to L-selectin was observed. Although solubility differences may account in part for the differences in neutrophil responsiveness to LPS from various bacteria species, L-selectin has been demonstrated to bind specifically to O-linked carbohydrates [26] and alterations in the polysaccharide moiety of LPS may have additional effects on the activity of L-selectin as a receptor for LPS. We are currently examining the involvement of neutrophil receptors in mediating LPS effects reported here.

Like steroid hormones, LPS has the ability to induce synthesis of new mRNA for proteins. In addition, the partial solubility of LPS in lipid may confer the ability to penetrate the plasma membrane and bind to the mitogen-activated protein kinase family [27]. Interestingly, our respiratory burst data show that smooth LPS could initiate intracellular signals, but more slowly than rough LPS. Since movement of proteins (like G proteins) to the cell membrane is an important feature of priming of neutrophil function by LPS, an alteration in LPS structure may affect lipid permeability and thus the capacity for binding intracellular compartments to modify protein translocation within the cell. The extent of glycosylation of LPS may, like dephosphorylation or deacylation, affect the ability of the lipid A moiety of LPS to be effective as an inflammatory mediator.

Conditions associated with septic shock or acute respiratory distress syndrome are characterised by activation and accumulation of leukocytes in tissues. The observation that neutrophil responsiveness to various LPS species can be influenced by the polysaccharide moiety of LPS point to new features in the mechanism of action of LPS. Carbohydrate modification of LPS by bacteria may confer some degree of protection against induction of host responses which have

important implications for the regulation of neutrophil effector function during the inflammatory response.

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